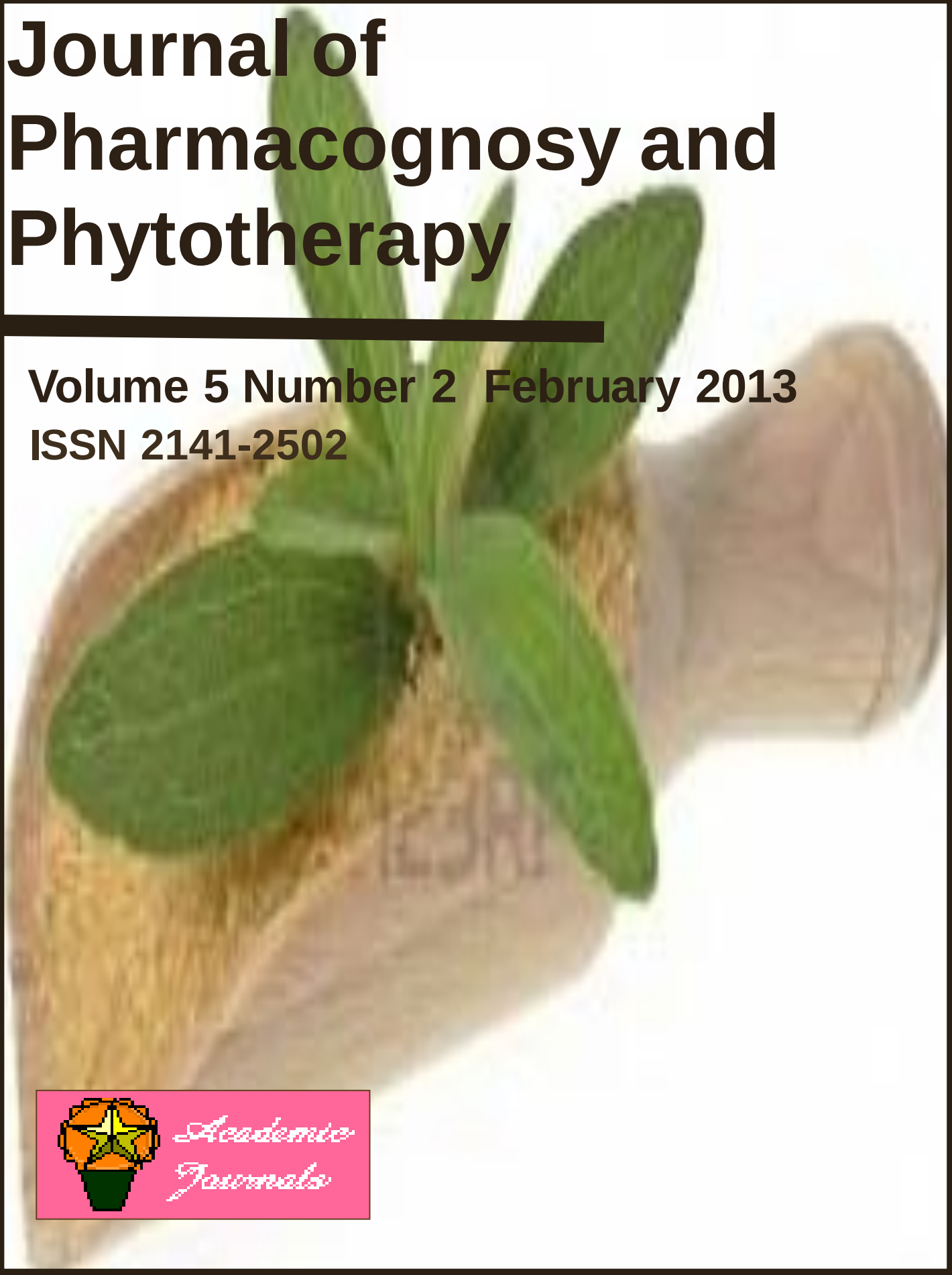
Journal of Pharmacognosy and Phytotherapy

Volume 5 Number 2 February 2013
ISSN 2141-2502



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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Journal of Pharmacognosy and Phytotherapy

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Available online at http://www.academicjournals.org/jpp
DOI: 10.5897/JPP2013. 0258
ISSN 2141-2502 ©2013 Academic Journals

Review

Plant tissue culture of *Stevia rebaudiana* (Bertoni): A review

S. S. Pande* and Priyanka Gupta

Rajiv Gandhi Biotechnology Centre, Rashtrasant Tukdoji Maharaj Nagpur University, L. I. T. Premises, Nagpur-440 033 (M.S.), India.

Accepted 11 February, 2013

Plant tissue culture is a technique used for *in vitro* regeneration of plants. It relies on maintaining plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells for an extended period of time or regenerated into whole plants. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture. This review paper outlines the work done on *Stevia rebaudiana,* a herbaceous perennial plant of the Asteraceae family. It is gaining lot of importance for the production of diterpene glycosides which are non-nutritive, non-toxic, high-potency sweeteners, and may substitute sucrose as well as other synthetic sweeteners, being 300 times sweeter than sucrose. The taxonomy, botanical description of the plant, its distribution and uses, are discussed in this paper. Various tissue culture methods to get mass propagation of *S. rebaudiana* are reviewed.

Key words: *Stevia rebaudiana,* asteraceae, tissue culture.

INTRODUCTION

Plant tissue culture is a science of growing plant cells, tissues or organs isolated from the mother plant. It includes techniques and methods used to research into many botanical disciplines and have several practical objectives. It is an experimental technique through which mass of cell is produced from the explants tissue. The callus produced can be utilized directly to regenerate plantlets or to extract or to manipulate some primary and secondary metabolites. Callus culture and suspension culture are the basic technique used to produce the desired metabolites of plants (Vyas and Dixit, 1999).

The plant and tissue cultures have been enabled to increase the knowledge in many areas including differentiation, cell division, cell nutrition and cell preservation but now, cells are cultivated *in vitro* in bulk or as clone from single cells to grow whole plants from isolated meristem, then induce callus and develop complete plantlets by organogenesis or by embryogenesis. The research needs are based on the elements of scientific progress and development of new techniques, which either enables enables more critical experiment to be undertaken, or rendering easy accessibility to complicated problems through experimental studies (Mantell and Smith, 1983; Evans et al., 1983).

Need for a biotechnological approach

Since the world population is increasing rapidly, there is extreme pressure on the available cultivatable land to produce food and fulfill the needs. Therefore, for other uses such as production of pharmaceuticals and chemicals from plants, the available land should be used effectively. The development of micropropagation methods for a number of medicinal plant species has been already reported and needs to be adopted (Naik, 1998). Plants are valuable source of a wide range of secondary metabolites which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides and food additives. Over 80% of the approximately 30,000 known natural products are of plant origin (Phillipson, 1990; Balandrin and Klocke, 1988).

Through plant tissue culture, the totipotent characteristics of plant can be used for the *in vitro* regeneration of plant. The great enthusiasms of biotechnologists are seen

*Corresponding author. E-mail: director.rgbc@gmail.com. Tel: +91-712-2552080.

in the potential use of cell culture in the production of valuable secondary products. Plant tissue culture is a noble approach to obtain their substances in large scale. In the present scenario, it is an effective and efficient procedure for converting less medicinally important plant metabolites to a valuable product. Many companies in India and abroad are showing interest in this direction.

Botanical description of *Stevia rebaudiana*

S. rebaudiana (Bertoni) is a herbaceous perennial plant of the Asteraceae family. It has an alternate leaf arrangement and herbaceous growth habit (Singh and Rao, 2005). Leaves are small, sessile, lanceolate to oblanceolate, oblong, serated above the middle and somewhat folded upwards. The leaf has a pleasantly sweet, refreshing taste that can linger in the mouth for hours. The material contains the sweet components, surrounded by the bitter components in the veins (Maiti and Purohit, 2008). The inflorescence is loosely paniculated with the heads appearing opposite the bracts in irregular sympodial cymes. The flowers are small (15 to 17 mm) and white (Marsolais et al., 1998; Dwivedi, 1999) with pale purple throat corollas.

Stevia is self-incompatible (Miyagawa et al., 1986; Chalapathi, 1997) and probably insect pollinated (Oddone, 1997). Since *Stevia* is self incompatible, seed collected from an individual plant would represent a half-sib family. *Stevia* is a short-day plant that flowers from January to March in the Southern hemisphere and from September to December in the Northern hemisphere. Seeds are contained in slender achenes about 3 mm in length. Each achene has about 20 persistent pappus bristles (Goettemoeller and Ching, 1999). Fertile seeds are usually dark coloured, whereas infertile seeds are usually pale or clear (Felippe, 1978; Monteiro, 1980; Oddone, 1997, 1999; Goettemoeller and Ching, 1999).

Distribution of *S. rebaudiana*

S. rebaudiana originated in the highland regions of Northeastern Paraguay (on the Brazilian border), between latitudes 23° and 24°S, where the unique sweetening power of its leaves and its medicinal properties have been known by the local Guarani Indians many hundreds of years (Chan et al., 1998; Melis, 1999; Jeppesen et al., 2002, 2003; Srimaroeng et al., 2005). The Guarani Indians called the plant ‘‘kaa he-he’’, which translates as ‘‘sweet herb’’, and used it as sweetener for their green herbal tea ‘‘mate’’, and as a flavor enhancer (Soejarto et al., 1982). In the native state, it grows on the edges of marshes or in grassland communities on soils with shallow water tables (Shock, 1982). It is indigenous to the Rio Monday Valley of the Amambay mountain region at altitudes between 200 and 500 m. The climate can be considered as semi-humid subtropical, with temperatures ranging from -6 to 43°C, with an average of 23°C, and rainfall ranging from 1500 to 1800 mm per annum. By now, the crop has been introduced to many countries, including Brazil, Korea, Mexico, the United States of America, Indonesia, Tanzania, Canada and India (Lee et al., 1979; Donalisio et al., 1982; Shock, 1982; Goenadi, 1983; Saxena and Ming, 1988; Brandle and Rosa, 1992; Fors, 1995).

Glycosides in *S. rebaudiana*

Eight diterpene glycosides with sweetening properties have been identified in leaf tissues of *Stevia*. These are synthesized, at least in the initial stages, using the same pathway as gibberellic acid, an important plant hormone (Singh and Rao, 2005). The two main glycosides are stevioside, traditionally 5 to 10% of the dry weight of the leaves, and rebaudioside-A (Reb-A), 2 to 4%; these are the sweetest compounds. There are also other related compounds including minor glycosides, such as rebaudioside-B, rebaudioside-C (1 to 2%), rebaudioside-D, rebaudioside-E, rebaudioside-F, dulcoside-A, dulcoside-C and steviolbioside, as well as flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids and some essential oils (Erik et al., 1956; Erich et al., 1961; Harry et al., 1956; Hiroshi et al., 1976; Masur et al., 1977; Yohei and Masataka, 1978; Rajbhandari and Roberts, 1983; Makapugay et al., 1984; Crammer and Ikan, 1986; Kinghorn, 1987; Tsanava et al., 1989; Shaffert and Chebotar, 1994; Putieva and Saatov, 1997; Dzyuba, 1998; Dacome et al., 2005; Sekaran et al., 2007). The stevioside, rebaudioside-A, rebaudioside-B, rebaudioside-C, rebaudioside-D, rebaudioside-E, dulcoside-A and steviolbioside are 250 to 300, 350 to 450, 300 to 350, 50 to 120, 200 to 300, 520 to 300, 50 to 120 and 100 to 125 times sweeter than sucrose, respectively (Crammer and Ikan, 1986; Yadav et al., 2011).

Rebaudioside-A

Among the components of *Stevia*, one, called rebaudioside-A, is of particular interest because it has the most desirable flavour profile (DuBois, 2000). Stevioside traditionally makes up the majority of the sweetener (60 to 70% of the total glycosides content) and is assessed as being 110 to 270 times sweeter than sugar. It is also responsible for the bitter aftertaste, sometimes reported as a ‘‘licorice’’ taste. As well as sweetness, stevioside may have a lingering effect or certain degree of pungency, which is not appreciated by the majority of people, and which reduces its acceptability. Rebaudioside-A is usually present as 30 to 40% of total sweetener and has the sweetest taste, assessed as 180

to 400 times sweeter than sugar, with no bitter aftertaste (licorice taste or lingering effect). The ratio of rebaudioside-A to stevioside is the accepted measure of sweetness quality; the more rebaudioside-A, the better the quality (Yadav et al., 2011). The yield of sweetening compounds in leaf tissue can vary according to the method of propagation (Tamura et al., 1984a), day length (Metivier and Viana, 1979) and agronomic practices (Shock, 1982). Unlike many low-calorie sweeteners, stevioside is stable at high temperatures (100°C) and over a range of pH values (Kinghorn and Soejarto, 1985). It is also non-calorific, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986).

Glycoside content in different plant parts

Sekaran et al. (2007) reported that individual tissues of *Stevia* appear to differ significantly in the stevioside content declining in order: leaves > shoots > roots > flowers. The fact that the highest stevioside content is found in the leaves suggests that they serve as the main tissue for both synthesis and primary accumulation of stevioside compounds.

Importance of *Stevia* tissue culture

Stevia is used to produce non-caloric sweetener which are natural alternatives to the synthetic sweetening agents. They do not metabolize in the human body (Randi, 1980), making *Stevia* safe for those who need to control their blood sugar level. With the current demand for food supplements having low carbohydrate, minimum calorie and low sugar content, the *Stevia* plant and its extracts have proven to be the ultimate choice. The health Benefits of *Stevia* are evident from the fact that they are approved as a dietary supplement by the Food and Drug Administration (FDA). In addition to its non-caloric sweetening properties, *S. rebaudiana* has many medicinally important properties. It is used for the treatment of various conditions such as cancer (Yasukawa et al., 2002), diabetes (Lailerd et al., 2004), obesity, cavities, hypertension (Dyrskog et al., 2005), fatigue, depression, and yeast infection. It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti-viral, anti-inflammatory, anti-bacterial properties and increases urination function of the body. It has been found to be non toxic, non addictive, non carcinogenic, non mutagenic, non teratogenic and is devoid of genotoxic effect. It does not affect blood sugar level, hence safe for diabetics (Alan, 2002; Mogra and Dashora, 2009). Hence, it is of large industrial and therapeutical value.

To exploit industrial application of *Stevia*, its large scale production is needed. Seeds of *Stevia* show a very low germination percentage (Felippe and Lucas, 1971; Monteiro, 1980; Toffler and Orio, 1981), and vegetative propagation through cuttings is limited by the small number of individuals (Sakaguchi and Kan, 1982). Tissue culture is the only rapid process for the mass propagation of *Stevia*.

PLANT TISSUE CULTURE OF *S. REBAUDIANA*

Researchers had tried to develop the plant through different tissue culture techniques, some of which researches had reviewed as the following.

In vitro clonal propagation of *S. rebaudiana* by stem-tip culture

Tamura et al. (1984) reported *in vitro* clonal propagation of *S. rebaudiana* by stem-tip culture. The clonal propagation was established by culturing stem-tips with a few leaf primordia on Linsmaier and Skoog (LS) agar medium (Linsmaier and Skoog, 1967) supplemented with a high concentration (10 mg/L) of kinetin. Numbers of shoots yielded from a single shoot tip in 80 days varied from 50 to 100. It was reported that the ability to form multiple shoots was dependent on the size of excised stem-tip and/or the number of leaf primordia. Anatomical examination had suggested that these multiple shoots originated from a number of adventitious buds formed on the margin of the leaf. It was estimated by these researchers that more than one million shoots could be obtained from an original stem-tip within 12 months. Successful transplantation to the tune of 83% in the soil was achieved.

Recently, Das et al. (2011) also reported micropropagation of *S. rebaudiana* through shoot tip culture. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2 mg/L kinetin was found to be best for multiple shoot proliferation, resulting in more than 11 shoots from a single shoot tip explants within 35 days of culture. For root induction, they found that MS media without growth regulators worked dynamically whereas when it was supplemented with indole-3-acetic acid (IAA) and benzyl adenine (BA), it had an adverse effect on root induction. They also performed peroxidase assay along with Inter-simple sequence repeat (ISSR) fingerprinting to confirm the genetic clonality of *in vitro* generated propagules. The above studies shows that micropropagation of *S. rebaudiana* can be achieved through shoot tip culture in agar medium containing high concentration of kinetin.

In vitro multiple shoot induction from nodal explants of *S. rebaudiana*

Yang et al. (1981) reported the *in vitro* multiple shoot induction from nodal explants of *S. rebaudiana*. They cultured

excised nodal segments in MS medium supplemented with various cytokinins at varying concentrations (1 to 10 mg/L). The highest axillary shoot proliferation was reported in medium containing benzyl adenine (2 mg/L), kinetin (10 mg/L) and N6-(2-Isopentenyl)-adenine (10 mg/L). For rooting, they employed naphthalene acetic acid (NAA) at varying concentration of 1 to 10 mg/L and it stimulated both percentage of cuttings with root formation and the number of roots per shoot.

Hwang (2006) reported the induction of adventitious shoots from nodal explants of field-grown plants on four basal media supplemented with various combinations of auxins and cytokinins. The best performance (23.4 ± 2.1 shoots per explant) was obtained on MS medium supplemented with 2 mg/L IAA and 0.5 mg/L kinetin (Kn). The *in vitro* regenerated shoots were transferred to MS medium supplemented with 3% sucrose and 2 mg/L Indole-3-butyric acid (IBA) for root induction. The rooted plants were hardened successfully and acclimatized to soil with 98.4% survival rate. Stevioside contents in the regenerated plants following transplantation was estimated to be 10.68 mg/g dry weight which was similar with that of mother plants (12.01 mg/g dw).

Ahmed et al. (2007) had also reported *in vitro* multiple shoot induction from nodal explants of *S. rebaudiana* through axillary shoot proliferation. The explants were cultured in MS medium supplemented with various combinations of BA and Kn. The axillary shoot proliferation was reported maximum on MS medium with 1.5 mg/L BA + 0.5 mg/L Kn. For rooting, different concentrations of IBA, NAA and IAA were used, and highest rooting percentage (97.66%) was recorded on MS medium with 0.1 mg/L IAA.

Rafiq et al. (2007) also reported *in vitro* propagation of *S. rebaudiana* through nodular stem sections. Maximum shoot formation was reported on MS media containing 2 mg/L BA. In contrast, 0.5 mg/L was found to cause the maximum root formation in nodular stem sections of *S. rebaudiana*. Various biochemical attributes (carbohydrates, proteins) in the leaves were also measured by these investigators and they compared it with leaf extract of Egyptian cultivar, with no significant difference in quality.

Alhady (2011) reported the micropropagation using stem node segment obtained from 2 years old plant. According to his report, the type of cytokinin was the most important factor affecting shoot multiplication. Cytokinin BA individually or in combination with kinetin at varying concentration was studied. The highest shoot multiplication rate was obtained on medium supplemented with BA. Increasing BA concentration promoted shoot multiplication. The maximum number of proliferated shoots was reported on MS medium supplemented with 2.0 mg/L BA + 0.5 mg/L Kn. However, medium supplemented with kinetin resulted in elongated shoots. For root induction, different concentrations of IBA and NAA were assayed. IBA showed to be more significant and effective for rooting than NAA in all concentrations used. The maximum root induction (100%) was observed on medium supplemented with 1.0 or 2.0 mg/L IBA. The above examples suggest that the use of cytokinins BA with kinetin promotes axillary shoot proliferation from stem node segment explants.

Establishment of callus culture in *S. rebaudiana*

Uddin et al. (2006) reported the establishment of callus culture in *S. rebaudiana.* Leaf, nodal and inter-nodal segments of the selected herb as explants were cultured on MS medium containing 2,4-D (2-4-Dichlorophenoxyacetic acid) at 2, 3, 4 and 5 mg/L. They observed that inter-nodal segments initiated callus earlier than node and leaf. It was reported that the highest amount of callus was found in MS medium with 3.0 mg/L 2,4-D, and MS medium with 5.0 mg/L 2,4-D gave the poorest callus.

Gupta et al. (2010) developed a protocol for callus induction and multiplication, they cultured nodal, leaf and root explants on MS medium with different concentrations of plant hormone like IBA, kinetin, NAA, 2,4-D, and NAA in combination with 2,4-D. Callusing was 100% from leaf explants cultured on combination of NAA and 2,4-D after three weeks while with 2,4-D, only 10% callusing was observed. The morphology of the calli formed from different explants was examined, and it was found that the calli obtained from leaf and root explants were shiny green while with nodal explants it was hard and brown. Investigators concluded that leaf explants could serve as a best planting material for callus production, and 0.75 mg/L NAA with 1 mg/L 2,4-D could produce maximum amount of callus within short time period. It can be seen, from the above examples, that 2,4-D individually or in combination with NAA enhances callusing in leaf and intermodal segments.

Establishment of suspension culture in *S. rebaudiana*

Ferreira et al. (1988) reported a method for suspension culture in *S. rebaudiana*. Suspension cultures was composed of isolated cells and cellular aggregates (5 to 100 cells) which were obtained in 20 to 30 days by using friable callus as the initial inoculum in liquid media with BA (0.5 mg/L) + 2,4-D (1.0 mg/L), and periodic filtering (100 to 500 µm sieves) with 6 to 7 days interval between subcultures. Cultures derived from actively growing calli are mainly diploid (2n = 22) whereas those derived from senescent calli showed a wide variation in chromosome number (55 to 200). Stock cell suspensions which had been maintained for 3 years were plated on basal LS agar medium with BA (0.5 mg/L) + 2,4-D (0.5 mg/L) to form callus.

Calli originating from predominantly 2n cell suspensions

when transferred to medium with Kn (2.0 mg/L) + NAA (0.02 mg/L) were able to form buds. Shoot elongation and further rooting of isolated shoots was better on LS medium devoid of growth regulators. Variation in rooting capacity, plant vigor, morphological characters and chromosome number was found amongst regenerated plants.

More research is required in this area since rapid production of cell suspensions with high growth rate will allow maintenance of genetically stable cells and can be used to obtain improved plants or stable cell lines capable of *in vitro* production of the sweetening principles.

Somatic embryogenesis in *S. rebaudiana*

Filho et al. (1993) reported the somatic embryogenesis in *S. rebaudiana.* To investigate the influence of growth regulators on the induction of somatic embryogenesis, leaf explants were inoculated on basal medium containing different concentrations of 2,4-D and BA under a high concentration of sucrose (120 g/L). The explants were incubated for 21 days in the dark, 7 days in light, under a photoperiod of 16 h, followed by more 7 days in the dark. Combination of 10 or 25 mM 2,4-D and 1 mM BA were found to be effective for somatic embryogenesis. The number of somatic embryos was recorded with aid of a stereomicroscope. Somatic embryos failed to mature and developed roots but not shoots on a MS medium without growth regulators, as reported.

Again, Filho and Hattori (1997) reported the somatic embryogenesis in *S. rebaudiana* but this time, they employed explants floret as explants. Explants were cultured on MS medium supplemented with 2,4-D (9.05 and 18.10 mM) and kinetin (0 to 9.29 mM), and the cultures were kept at 28°C under dark. Formation of embryogenic callus was first observed 10 days after inoculation. The embryogenic callus was characterized by a light green or light yellow color, compact structure and presence of globular somatic embryos on its surface. Further investigators reported that on 9.05 Mm 2,4-D supplemented medium, maximum embryogenic callus formation occurred in medium without kinetin. On 18.10 mM 2,4-D supplemented medium, the best treatment was 2.32 mM kinetin. Whereas at 4.52 mM 2,4-D, embryogenic callus formation was limited to only one explant (on medium without kinetin). Embryogenic callus started at the base of the corolla and ovary. Histological sectioning was performed which showed a fibrillar network on the surface of somatic pro-embryos. No vascular connections between the embryo structures and the initial explant were observed. A unicellular origin of the somatic embryos was proposed by the researchers. The development of protocols for regeneration of *S. rebaudiana* via somatic embryogenesis is important, as this technique can be used in the clonal propagation of this plant.

Anther culture in *S. rebaudiana*

Anther culture is usually used to obtain haploid plants from which doubled haploids/homozygous plants can be developed through colchicine treatment in a short time and also in crops where self-incompatibility is the limiting factor for the development of homozygous plants or inbred lines. Flachsland et al. (1996) regenerated plants from anthers cultured *in vitro* under defined conditions. Anthers (containing uni-nucleate microspores) were induced to form callus when aseptically cultured on MS liquid medium supplemented with 0.1 to 1 mg/L benzylaminopurine (BAP). Regeneration of shoots was readily achieved by transferring pieces of callus to fresh solid medium with the same composition. Shoots were induced to form roots upon transfer to medium with 0.1 mg/L NAA. Plantlets were successfully potted, but cytological studies of root tips from regenerated plants revealed a normal diploid number of chromosomes (2n = 22). The study implies that somatic cells of the anther wall respond to the high BAP concentration in the medium (Yadav et al., 2011). Anther culture can be very useful in developing homozygous plants with high steviosides and rebaudioside contents, and more investigations are required in this area.

Regeneration protocols of *S. rebaudiana*

Many researchers had tried to develop regeneration protocols for *S. rebaudiana*. Few of such recent investigations are reviewed.

Patel and Shah (2009) reported the regeneration of *S. rebaudiana* through callus culture. In this investigation, callus induction and multiplication medium was standardized from nodal as well as leaf segments. For callus induction, explants were cultured on MS medium, with varying concentration of BA and NAA. 2.0 mg/L BA + 2.0 mg/L NAA was reported to be the best for callus induction, and higher regeneration frequency was also noticed with this combination. Regenerated plants were rooted better on ¼ MS strength supplemented with 0.1 mg/L IBA and they were hardened successfully in tera care medium with 63% survival rate. The developed protocol can be utilized for mass planting of material on large scale, independent of season, that is external environmental conditions.

Also, Moktaduzzaman and Rahman. (2009) reported the regeneration of *S. rebaudiana*, and they analyzed the somaclonal variation among regenerated plants by random amplification polymorphic DNA (RAPD), since identification of possible somaclonal variants is considered to be very useful for quality control in plant tissue culture and in the introduction of variants. In their experiment, the leaf segments were cultured on MS medium supplemented with the different concentrations and combinations of NAA + BA and 2,4-D + BA. It was

reported that 1.5 mg/L NAA with 1.0 mg/L BA was the best for callus induction (91.67%) which also produced highest fresh weight (621.7 mg) and dry weight (79.00 mg) of callus. Calli were then transferred to MS medium with different concentrations and combinations of BA and NAA with control. The highest number of shoots (2.17) and the highest average length of the shoot (3.22 cm) per culture were reported at 1.8 mg/L of BA with 0.12 mg/L of NAA.

For root induction, same concentration of IBA and NAA was used. But 1.00 mg L^{-1} NAA produced highest number of roots and highest length of roots per culture, reportedly. The regenerated plantlets were successfully hardened in 75% soil and 25% sand mixture, and finally transferred into the field. Apparently, researchers examined somaclonal variations among regenerated plants along with mother plant by RAPD using A03 primer. Bands generated through random amplification polymorphic DNA-polymerase chain reaction (RAPD-PCR) were scored according to whether they were present or absent to determine the extent of somaclonal variation. The estimation of genetic similarity coefficient based on RAPD band-sharing data analyzed indicated that some regenerated plants were 100% similar to the mother plants and some were 71, 57 or 14% similar, which may be due to variation in *in vitro* condition.

In the same year, Sairkar et al. (2009) reported standardization of *in vitro* culture technique to explore potential of *S. rebaudiana* for micro-propagation and callus culture. Nodal segments of the selected herb as explants were cultured for micro-propagation on MS medium containing 0.1 mg/L N6-benzyl amino purine for shoot initiation. Maximum plantlets (83.2 ± 0.445033) were found in MS medium treated with 3.5 mg/L N6-benzyl amino purine at multiplication stage. Combination of 2 mg/L 2-4-Dichlorophenoxyacetic acid + 1 mg/L kinetin was reported to give best result of callusing. Higher regeneration of plantlets (3.8 plantlets/calli) was reported in MS medium with 5 mg/L BA + 1 mg/L NA. Highest rooting average (11.1 ± 0.264052) was recorded on ½ MS medium with 100 mg/L activated charcoal. The rooted plantlets were hardened in 1:1:1 ratio of sand: soil: vermicompost, and successfully established in soil.

Anbazhagan et al. (2010) also reported the mass propagation of *S. rebaudiana.* Shoot tip, nodal segment and leaf were used as explants and they were cultured on MS medium supplemented with different concentrations of BA, Kn and IAA both in individual and in combined form for shoot inductions, and the best results were obtained from MS medium supplemented with BA + IAA at the concentrations of 1.0 and 0.5 mg/L, respectively. Among the explants used, shoot tip stood first in inducing shoot development. Best root formation of *in vitro* developed shoots could be achieved on half-strength Nitsch (N6) medium supplemented with IAA at concentration 1.0 mg/L. The *in vitro* developed plantlets were transferred to pot and they were grown in greenhouse for hardening, and finally they were planted in the open field. Around 82% of plants were successfully established in natural field condition as reported.

Recently, Singh et al. (2011) also reported the plant regeneration under *in vitro* conditions. Callus was efficiently induced when leaf segments were cultured on MS medium supplemented with 1.0 mg/L 2,4-D + 1.0 mg/L kinetin. MS medium + 0.5 mg/L BAP + 0.3 mg/L NAA was reported best for root differentiation, and shoot differentiation was best observed in MS medium + 0.5 mg/L BAP + 0.1 mg/L NAA. These investigators also studied the metabolic changes during differentiation in callus cultures. It was reported that metabolites like starch, total soluble sugars and total phenols decreased while total soluble proteins increased in callus culture during the process of root and shoot differentiation.

From the above cited experiments, it can be concluded that the use of BA and NAA promotes callus induction and shoot differentiation whereas NAA and IBA enhances the root induction. Hence the above stated combinations of growth regulators can confer better results for regeneration of this sweet herb.

CONCLUSION AND RECOMMENDATIONS

In vitro propagation can become an important alternative to conventional propagation and breeding procedures for *S. rebaudiana* which is both an industrially and medicinally important herb. The above reviewed studies show potential for use of tissue culture methods to get a rapid mass propagation of *S. rebaudiana*. The explants - and plant growth regulators levels have significant impact on accelerated micropropagation of *Stevia* to regenerate, genetically true to the type propagules. In most cases, growth regulators like BAP and NAA were found to be essential for growth and multiple shoot formation. Although results are promising, there is a scope of improvement, further research and development need to be carried out to improve this herb's potential as a crop by developing improved varieties with commercially significant yield.

Protocols for regeneration through somatic embryogenesis and anther culture need to be developed as it can help in producing true to the type and homozygous plants with improved quality. Improved genotypes with a high content of rebaudioside-A need to be developed and propagated as it has most desirable flavor characteristics. Further investigations are required to develop techniques for the commercial production of desirable glycosides of *Stevia* in a suitable bioreactor system.

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Journal of Pharmacognosy and Phytotherapy Vol. 5(2), pp. 34-37, February 2013
Available online at http://www.academicjournals.org/jpp
DOI: 10.5897/JPP12.031
ISSN 2141-2502 ©2013 Academic Journals

Full Length Research Paper

Investigative study on the angiotensin converting enzyme (ACE) inhibiting properties of the terpenoid extract of *Crataegus monogyna* using *in silico* models

D. L. Farrugia1*, C. M. Shoemake1, E. Attard2, L. M. Azzopardi1 and S. J. Mifsud1

1Department of Pharmacy, Faculty of Medicine and Surgery, University of Malta, Msida, Malta.
2Division of Rural Sciences and Food Systems, Institute of Earth Systems, University of Malta, Msida, Malta.

Accepted 11 February, 2013

Crataegus monogyna **is mainly used in the treatment of cardiac and circulatory system disorders.** ***In vitro*** **and clinical studies are indicative of the fact that the hydroethanolic extract of** ***C. monogyna*** **has angiotensin converting enzyme (ACE) inhibitory activity. This study sought to support these claims through the use of** ***in silico*** **modelling techniques. Possible binding conformations for β-amyrin, oleanolic acid and ursolic acid were generated using captopril, as well as enalaprilat and lisinopril, as template ligands. The ligand binding affinity (LBA) of each was calculated and the best binding conformation of each triterpene was established. Results indicate that these naturally occuring terpenes possess** ***in silico*** **predicted ligand binding affinities that are superior to both the small molecule captopril and the larger molecules enalaprilat and lisinopril.**

Key words: *Crataegus monogyna*, hydroethanolic extract, angiotensin converting enzyme (ACE) inhibition, *in silico*.

INTRODUCTION

Hypertension is recognised as being one of the most preventable causes of premature morbidity and mortality. The World Health Organization (WHO) statistics indicate that hypertension affects approximately 40% of all individuals aged 25 years and over. It is prevalent worldwide in both developed and developing countries (WHO, 2012). The majority of hypertensive patients currently rely on the use of angiotensin converting enzyme (ACE) inhibitors for management of their condition (Sweileh et al., 2009).

In silico models are of value in that they allow virtual screening for desirable properties and further optimization of identified lead candidate molecules, thus drastically lowering financial and time requirements for the chemical synthesis and biological testing of promising leads. They also minimise animal testing; a factor that should not be considered superficial in the highly regulated and ethically conscious scenario in which contemporary drug discovery operates (Kapetanovic, 2008).

In vitro and clinical studies have indicated that the triterpenic extract of *Crataegus monogyna* is capable of exerting an inhibitory effect on the ACE (Attard and Attard, 2006). Subsequent to this work, we hereby report the results of a static *in silico* investigation of the ACE and of the molecular and conformational bases for the ACE inhibitory effect exerted by the terpenoid extract of *C. monogyna*, which to our knowledge has as yet not been described.

*Corresponding author. E-mail: dfar0021@um.edu.mt. Tel: +35679496191.

MATERIALS AND METHODS

Protein data bank (PDB) deposition selection

Three Protein data bank (Bernstein et al., 1977) depositions were selected as templates for this study. These were PDB IDs 1UZF (Natesh et al., 2004), 1UZE (Natesh et al., 2004) and 1O86 (Natesh et al., 2003), describing the bound co-ordinates of testicular ACE and the small molecules captopril (resolution 2.00 Å), enalaprilat (resolution 1.82 Å) and lisinopril (resolution 2.00 Å).

PDB deposition visualisation and modelling

Molecular visualisation and modelling were carried out using SYBYL® (SYBYL 7.3, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.) (SYBYL 7.3, Tripos International, Cartera™). The selected PDB depositions were treated identically during this phase of the study. Specifically, each crystallographic deposition 1UZF, 1UZE and 1O86 was read into SYBYL® (SYBYL 7.3, Tripos International, Cartera™) with precautions being taken to preserve the bound co-ordinates and consequently the bioactive conformation of each. All moeities considered as superfluous to binding were edited. This means that in the case of 1UZF, two chlorine atoms, 2 N-acetylglucosamine molecules and all water molecules lying at a distance ≤ 5 Å were removed. In the cases of 1UZE and 1O86, two chlorine atoms and two glycine molecules were removed. The result of this editing process consequently was *holo*-ACE bound to captopril, enalaprilat and lisinopril, respectively with water molecules at a radius ≤ 5 Å being retained.

Retention of these water molecules was carried out on the premise that crystallographic data was suggestive of the fact that their proximity to the bound small molecules and the ligand binding pocket (LBP) could give rise to a situation in which bound ligands could be stabilised within the LBP through the formation of water bridges. This editing process was performed in preparation for molecular dynamics studies which will be carried out during subsequent stages of this study.

The small molecules captopril, enalaprilat and lisinopril were subsequently extracted, using SYBYL® (SYBYL 7.3, Tripos International, Cartera™), from their respective ACE ligand binding pockets (ACE-LBP). Each small molecule was saved in PDB and mol2 formats while the *apo*-ACE with the retained water molecules (n = 143, 455 and 569, respectively) were saved in pdb format. The choice of file format in which to save each moeity was a function of software requirements of subsequent phases of the study. Specifically, ligand binding affinity (LBA) and *de novo* design were carried out using the algorithms of Wang and co-workers in SCORE® (Wang et al., 1998) and LigBuilder® (Wang et al., 2000), respectively. These require that small molecules be saved in mol2 format and that the protein receptor be saved in pdb format. VMD® (Humphrey et al., 1996) was used for image generation and this required that all molecules be read in pdb format.

Analysis of the ligand binding pocket

The extracted bioactive conformations of the small molecules captopril, enalaprilat and lisinopril were used as probes in order to generate ACE-LBP maps of each bound conformation of the ACE. This was done using the pocket module of LigBuilder ® (Wang et al., 2000) which exploited the contacts forged between the small molecule and the ACE-LBP in each case to generate a 3D bond type specific map of the ACE-LBP as described in each deposition being studied. This process also generated proposed pharmacophores for each deposition. Elucidation of ligand specific LBP maps and pharmacophores was carried out based on the fact that it is known that receptor tertiary structure and LBP conformation is specific ligand driven. This process consequently served to highlight the conformational differences that the 3 ligand probes induced within the ACE-LBP. At the end of this process, consequently, 2 output files were generated for each deposition. These were:

1. The key_site_file depicting the key interaction sites between the amino acid side chains lining the ACE-LBP and the resident small molecule. By convention, hydrogen bond donor sites were represented in blue, hydrogen acceptor sites were represented in red, while hydrophobic sites were represented in cyan.
2. The pharmacophore_file depicting the proposed pharmacophoric model. Colour conventions were assigned in the same manner as described for the key_site_file.

Both files were saved in pdb format such that they could be later used in the visualization software VMD® (Humphrey et al., 1996).

Estimation of the ligand binding affinity (LBA) of the template ligands for the ACE receptor

The *apo*-ACE files which were generated when the selected pdb depositions were edited together, with their respective extracted small molecules saved in mol2 format, which were read into and processed in SCORE® (Wang et al., 1998). Through its static algorithm, the in silico provided LBA (pK_d) of each of the small molecules captopril, enalaprilat and lisinopril and was calculated for the respective cognate receptor.

Superimposition of the triterpene molecules onto the bound coordinates and 3D volume of the template ligands

In this part of the study, the bioactive conformations of the small molecules captopril, enalaprilat and lisinopril together with the 3D volumes which they occupy within their respective ACE-LBP conformations were used to guide β-amyrin, oleanolic acid and ursolic acid, all of which are constituents of C. monogyna, into the 3 conformations of the ACE-LBP. This was done in order to identify the highest affinity conformers for each molecule, and to utilise these during subsequent stages of the rational drug design process. Identification of the optimally binding conformations of the triterpenic small molecules was carried out using the *Similarity Suite* algorithm in SYBYL® (SYBYL 7.3, Tripos International, Cartera™). This algorithm facilitated initial positioning of each small molecule within the ACE-LBP based on the conformation of its cognate ligand. Subsequent to this process the triterpenic molecules were allowed conformational rotation within each ACE-LBP conformation. The 21 conformers for each molecule which exhibited optimal binding characteristics were selected and their *in silico* LBA (pK_d) was then quantified in SCORE® (Wang et al., 1998).

RESULTS

The *in silico* LBA (pK_d) of captopril, enalaprilat and lisinopril for their cognate receptor was predicted to be 5.36, 6.44 and 6.53, respectively. Significant differences in ACE-LBP occupation by captopril, enalaprilat and lisinopril could be seen when the LBP maps generated by

Table 1. The LBA (pK_d) of the 21 optimally binding conformers of each terpene ligand based on the three different template ligands employed in this study as calculated in SCORE®.

Captopril			Enalaprilat			Lisinopril		
β-amyrin	Oleanolic acid	Ursolic acid	β-amyrin	Oleanolic acid	Ursolic acid	β-amyrin	Oleanolic acid	Ursolic acid
6.56	6.95	6.74	7.03	7.04	7.26	6.98	6.78	7.37
6.56	6.95	6.74	7.00	7.13	7.23	7.05	6.66	7.34
6.30	7.04	6.82	7.24	7.14	7.35	6.81	7.19	7.41
6.55	6.62	6.68	7.10	7.21	7.21	7.09	6.93	6.95
6.56	6.70	6.69	7.04	7.17	7.15	7.11	6.94	6.85
6.62	6.64	7.18	7.15	7.13	7.59	7.00	7.00	6.96
6.74	6.68	7.14	7.18	7.20	7.29	6.64	7.04	7.02
6.63	6.94	6.53	7.03	7.20	7.19	6.89	6.99	7.30
6.60	6.99	6.94	6.94	7.19	7.36	6.62	6.89	7.34
7.25	6.53	7.27	7.30	7.41	7.25	6.53	7.35	6.85
6.57	6.94	7.30	7.25	7.05	7.31	7.25	6.59	7.27
6.67	6.89	7.24	7.20	7.42	6.93	6.88	6.87	7.08
6.61	6.64	7.29	7.20	7.43	7.42	6.89	6.90	7.05
6.64	6.69	7.69	6.69	7.44	7.43	6.94	6.90	7.17
6.56	6.98	7.84	6.67	7.46	7.26	6.94	6.52	7.07
6.47	6.36	7.11	6.68	7.25	7.08	6.55	6.52	6.62
6.47	6.94	7.79	6.77	7.29	7.21	6.74	6.37	6.81
6.47	6.47	7.15	7.05	7.11	7.20	6.39	6.73	6.63
6.82	6.61	7.41	6.80	7.02	6.67	6.68	6.74	6.80
6.86	6.17	6.76	6.77	7.05	6.58	6.74	6.11	6.55
6.56	6.95	6.83	7.03	7.04	7.26	6.98	6.78	7.37

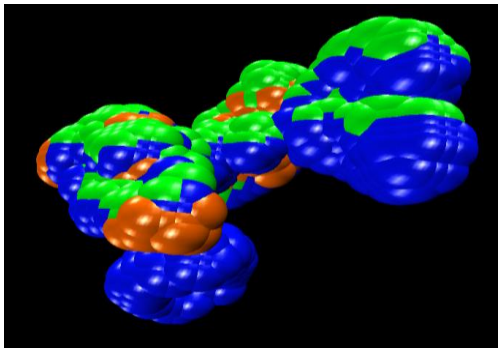

Figure 1. Key interaction sites of captopril (in orange), enalaprilat (in green) and lisinopril (in blue) rendered in VMD®.

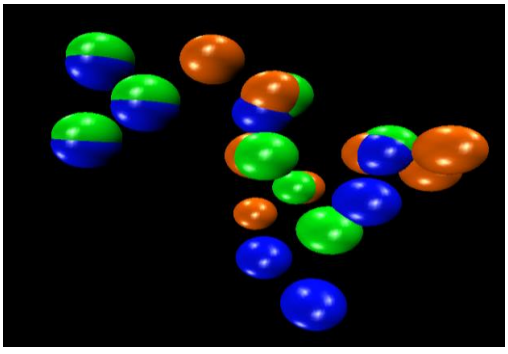

Figure 2. Proposed pharmacophores of captopril (in orange), enalaprilat (in green) and lisinopril (in blue) rendered in VMD®.

each molecule were compared (Figure 1). Similarly, the proposed pharmacophores in each case were also dissimilar (Figure 2). The LBAs of the 21 optimally binding conformers of β-amyrin, oleanolic acid and ursolic acid for the captopril-, enalaprilat- and lisinopril- bound conformations of the ACE were calculated and are shown in Table 1. The most salient finding in this case was that the predicted *in silico* LBAs of all 3 experimental triterpenes (pK_d 7.25, 7.46 and 7.84) exceeded those calculated for captopril, enalaprilat and lisinopril (pK_d 5.36, 6.44 and 6.53, respectively).

DISCUSSION

This *in silico* study further corroborates the hypothesis

of Attard and Attard (2006) that the triterpenic extract of *C. monogyna* has ACE inhibitory activity. All three molecules β-amyrin, oleanolic acid and ursolic acid exhibit binding affinities (7.25, 7.46 and 7.84, respectively) that are superior to those of captopril, enalaprilat and lisinopril (5.36. 6.44 and 6.53, respectively), implying a superior inhibitory activity when compared to the ACE inhibitors that are currently in widespread clinical use. Although this study utilised algorithms that were static, it was still possible to infer the importance of understanding the dynamic nature of both the ACE and its cognate small molecules. This is borne out by the fact that ACE-LBP maps and proposed pharmacophores were obtained that differed according to resident ligand. This was indicative of the fact that LBP conformation is essentially ligand driven. The fact that for example, ursolic acid had a LBA which was highest when captopril was used as a template may be taken to imply that ursolic acid adopts a captopril-like conformation within the ACE-LBP. A similar conclusion may be drawn for oleanolic acid whose best LBA was observed when its conformation within the ACE-LBP was modelled on that of enalaprilat. It was also interesting to note that in the case of β-amyrin, the *in silico* calculated LBA for its optimally binding conformation remained constant irrespective of which ACE inhibitor it was modelled on. This conformation of β-amyrin is consequently very interesting from a rational drug design point of view.

The results obtained from this study consequently open new avenues for the rational design of ACE inhibitors based on the triterpenic scaffold. A molecular dynamics simulation through which the dynamic interactions between the ACE and the ACE inhibitors captopril, enalaprilat and lisinopril may be compared with those between the ACE and the triterpenic molecules β-amyrin, oleanolic acid and ursolic acid represents the next step in this direction.

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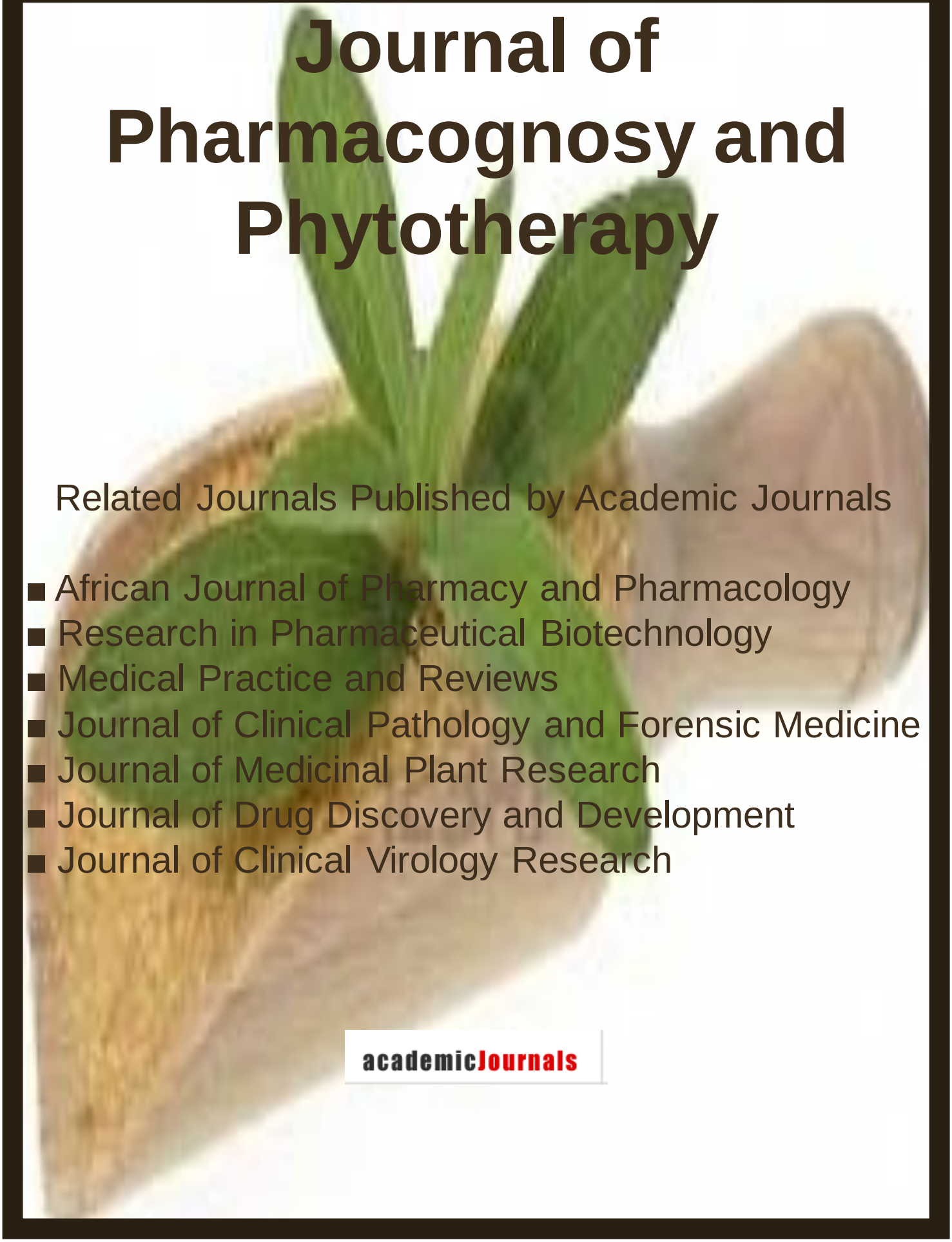

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