*Full Length Research Paper*

# Development of extraction protocol for phenolic compounds in musk tree seed (*Buchholzia coriacea*)

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**The impact of extraction methodology and polarity of extraction solvents on the assay of phenolic compounds was investigated using musk tree seed (*Buchholzia coriacea*) as a model substrate. The efficiency of four solvents (methanol, ethanol, acetone and ethyl acetate) at 20, 40, 60, 80 and 100% aqueous solution and five extraction methods (shaking, vortex mixing, stirring, sonication, sonication/vortexing and accelerated solvent extractor) in extracting the phenolics of *B. coriacea* were evaluated. Extracts were analyzed for phenolic content by high-performance liquid chromatography and Folin–Ciocalteu assays. Two major phenolic peaks (1 and 2) were isolated from the *B. coriacea* seed extract in the HPLC which were suspected to be lupeol and β-sitosterol. Methanol at 20% aqueous solution was found to be most efficient in extracting the total phenolic and HPLC peak 1 component of *B. coriacea* whereas acetone at 80% aqueous solution was more efficient in extracting peak 2 component. On the other hand Accelerated Solvent Extractor (ASE) extraction procedure was noted to be most efficient for extracting the total phenolics and HPLC peak 2 components while sonicator/vortexing was more efficient in extracting peak 1 component.**

**Key words:** Solvent, extraction, method, musk tree, phenolic.

## INTRODUCTION

Polyphenols are a large family of metabolic compounds that occur naturally in a wide variety of plant foods (Milner 1994; Luthria and Mukhopadyhyay, 2006). Recently, polyphenols have attracted a great deal of attention due to their roles as natural antioxidants. Antioxidants are believed to provide protective effects against oxidative damage diseases such as cancer, coronary heart disease and stroke. In fact epidemiological, clinical and laboratory studies suggest that consumption of fruits, vegetables and herbs containing polyphenols reduce the risk of chronic diseases such as coronary heart disease and cancer (Hertog, 1995; Rice-Evans et al., 1996; Middleton et al., 2000; Robbins 2003; Wang, 2013). According to Milner (1994) approximately 90% of all cancer cases correlate with environmental factors, including one's dietary habits, and one-third of all cancer

deaths in the United States are avoidable by changing dietary habits only. These discoveries have rapidly amplified the consumer awareness of the potential benefits of naturally occurring compounds from plants in health promotion and maintenance. One critical challenge in phytochemistry and polyphenolic studies is the identification and quantification of the phenolic compounds in different plant materials.

The structural diversity of phenolic compounds presents a significant challenge for developing a uniform methodology that is suitable for extraction of all phenolics or a specific class of phenolic compounds. The issue of developing a satisfactory extraction procedure is further complicated as phenolics are not uniformly distributed in plants at the tissue, cellular and subcellular levels. In addition, these compounds can be found in free, conjugated and polymeric forms or may coexist as complexes with carbohydrate, protein or other plant components. All of the above factors directly impact the solubility of phenolics in different solvents. In spite of all

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the above issues, simple extraction has often been overlooked. According to Rong and Zeyuan (2004), extraction method is critical to the recovery of antioxidant phytochemicals. The nature of both plant materials and the bioactive components should be considered in order to achieve good extraction efficiency. Extraction of phenolic compounds from plant materials is influenced by various parameters such as solvent polarity, particle size, extraction procedures and conditions. The impact of the extraction of phenolic compounds on the analysis has often been overlooked as substantial variations in the extraction procedures and solvents are documented (Antolovich et al., 2000; Naczk and Shahidi, 2004). Several solvents such as methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethyl formamide, dimethyl sulfoxide and their combinations have been used for the extraction of different classes of phenolic compounds (Majors, 1995; Vrhovsek and others, 2004; Parejo et al., 2004; Wu et al., 2004; Maatta-Riihinen et al., 2004). The extraction procedure is determined by the types of antioxidants to be extracted and whether the objective is quantitative or qualitative.

One interesting plant that promises to be a very good source of polyphenols is *B. coriacea*. *B. coriacea* also known as musk tree is an evergreen understorey tree of lowland rain forest, growing up to 20 m high. It is found in tropical regions –Nigeria, Ghana, Liberia and Cameroon etc. The fruit is about 5 inches long, resembling avocado pear, containing few large blackish seeds, about 1 inch long. The seeds are edible and taste peppery (Ajaiyeoba, 2000). Phenolics-Alkaloids, flavonoids, glucosinolates and sterols have been implicated in the members of this plant family which possess antimicrobial properties (Bombadelli et al., 1973; Ajaiyeoba et al., 2003). Ajaiyeoba et al. (2003) observed that fractions prepared from the methanol extract of the *B. coriacea* stem bark exhibited a high concentration dependent anti-bacterial and antifungal activity compared to the standard antibiotics, ampicillin and tioconazole.

The objectives of the present study were to develop the extraction protocols for the phenolic compounds in *B. coriacea* and quantify them.

## MATERIALS AND METHODS

The fruits of *B. coriacea* were purchased from Ishiagu in Ebonyi State, Nigeria. They were deseeded and the seeds were cleaned and the external hull of the seeds removed by hand. The seeds were then cut into an average size of 3 mm by 4 mm and dried under the sun. They were finally milled into powder (passing 0.5 mm screen) using a laboratory hammer mill (Thomas Wiley mill: ED-5: serial number790702, cat number3379-k30). The powder was then stored in cellophane and kept in a freezer.

## Comparison of the extraction efficiency of solvent composition

A systematic variation of different portions of two solvent mixtures (20:80, 40:60, 60:40 and 80:20 v/v) of methanol, acetone, ethanol, and ethyl acetate to water was used to compared the extraction efficiency of the phenolic compounds from *B. coriacea*. Also the efficiency of 100% (100:0) of each solvent was evaluated. The volume of the combined extract was adjusted to 25 ml with the corresponding extraction solvent and appropriate aliquots filtered through a 0.45 μm PVDF syringe filter prior to total phenolic and High Performance Liquid Chromatography (HPLC) assay. Duplicate extractions, Folin-Ciocalteu (FC) assays and HPLC analyses were carried out for each sample. All extractions were carried out with approximately 250 ± 1 mg of dried powdered flour of the *B. coriacea* (particle size < 0.5 mm) by using ultrasonic bath (sonication). The extraction efficiency was computed using the maximum yield produced by any of the solvents (as 100%) and calculating other solvents' efficiency by dividing this maximum yield with the individual solvent's yield as enunciated by Luthria and Mukhopadhyay (2006).

## Comparison of extraction procedures

The dried *B. coriacea* powdered sample was extracted with MeOH – $H_2O$ (20:80, v/v). Extractions were carried out using five different procedures namely, shaking, vortex mixing, stirring, sonication, and sonication-vortexing and accelerated solvent extractor. Extractions were carried out using the same solid to solvent ratio and solvent mixture as described by Justesen (2000). For each, approximately 250 ± 1 mg of the powdered sample was placed in a 16 x 125 mm screw-capped vial and 10 ml of the $CH_3OH$–$H_2O$ (20:80, v/v) solvent mixture added. In the shaker procedure, the vial was first inserted into a plastic centrifuge tube before placing inside a 250 ml conical flask, and then placed on a model G76 gyratory wrist shaker (New Brunswick scientific Co. Edison N.J. USA) at high speed (setting number 10) for 30 min. For sonication, the vials were placed in a sonicator bath at 40°C for 30 min. In combined sonication and vortexing, the vials were first vortex-mixed for about 1 min before the sonication and vortex-mixed (1 min) after sonication. Stirring was carried out by adding one 8 x 1.5 mm magnetic bar to each vial and placing the vial in a 100ml beaker on a lab – line multi-magnetic stirrer at ambient temperature and stirring for 30 min. Extraction with vortex mixing was performed by vortex mixing the vials for 2 min (three times) on a Vortex – Genie 2 scientific industry (Bohemia, N.Y. USA). After extraction with the different procedures, the mixture was centrifuged at low speed (10000 x g) for 10 min. the supernatant was transferred into a 25 ml volumetric flask. The residue was

resuspended in an additional 5 ml of $CH_3OH$ – $H_2O$ (20:80, v/v), gently mixed manually for 30 s and centrifuged for 5 min. The supernatant was combined with the first extract. The volume of combined supernatant was made up to 25 ml with extraction solvent and 2 ml aliquots of extracts were filtered through a 0.45 µl PVDF syringe filter for phenolic assay by the FC method and HPLC analysis. For each sample, extraction and analyses were carried out in duplicate.

Accelerated Solvent Extractor (ASE) (model ASE 200, Dionex Corporation, Sunnyvale, CA, USA) was also used. Aliquots of 250 ± 1 mg of dried powdered *B. coriacea* were placed in an 11 ml stainless-steel extraction cell. Two circular cellulose filters (size 1.983 mm, Dionex) were placed at the bottom of the extraction cell in order to prevent suspended particles from entering the collection vials. The remaining void volume in the cell was filled with diatomaceous earth. Both extraction cells and collection vials were arranged appropriately in the two designated carousels. Extractions were carried out with $CH_3OH$ – $H_2O$ (20:80, v/v) solvent mixture. Extractions were performed at 1000 psi (6.67MPa), with a 5 min equilibration time, a 10 min static time and a 90 s purge time for each extraction cycle. Four extraction cycles were performed for each sample. Only for comparison with the other extraction procedures, the static time was set to 5 min in order to complete the extraction per sample within 30min. the extractions were carried out at 40°C and a total of about 20 ml of solvent was obtained for the four extraction cycles with the flush volume set at 75%. The extracts were collected in 60 ml sample vials wrapped with aluminum foil and fitted with Teflon-coated rubber caps. Each extract was transferred to a 25 ml volumetric flask and the total volume was adjusted to 25 ml with the appropriate solvent mixture. Aliquots of the extracts were filtered through a 0.45 µl PVDF syringe filter prior to analysis of phenolics by FC assays and HPLC analyses were carried out for each sample. Also the extraction efficiency was computed using the maximum yield produced by any of the methods (as 100%) and calculating other methods' efficiency by dividing this maximum yield with the individual method's yield (Luthria and Mukhopadhyay, 2006).

## Determination of total phenolics (TP) by Folin-Ciocalteau (FC) assay

The TP content was determined using FC assay (Singleton, 1974) with garlic acid as a standard on a Varian Bio 50 UV spectrophotometer. The assay was carried out by pipetting 60 µl of the *B. coriacea* extract into an 8 ml vial. This was followed by addition of 4.74 ml of water. The mixture was vortexed mixed for 10 – 20 s and 300 µl of FC reagent were added. The mixture was vortexed mixed for an additional 10 – 20s, 900 µl of filtered 200 g/L sodium carbonate solution were added after 1 min and before 8 min of addition of the FC

reagent. This was recorded as time zero; the mixture was vortexed 20 – 30 s after the addition of sodium carbonate. After 2 h ± 3 min, at ambient temperature, the absorbance of the coloured reaction product was measured at 765 nm. A calibration curve was created using standard gallic acid solutions each time an analysis was run. The level of TP in the extract was calculated from the calibration curve. The results were expressed in mg of gallic acid equivalent per gram ($mgGAEg^{-1}$) of the dried *B. coriacea* flour.

## Determination of the phenolic compounds by HPLC

The samples (10 µl of the extract) were separated using a HPLC system (LCMS-QP8000, Shimadzu) coupled with a photodiode-array detector (DAD). A reverse-phase C18 lunar column (phenomenex, Torrance, CA, USA). The column was thermostatistically controlled at 40°C and the flow rate was set to 0.5 ml min-1. The mobile phase consisted of only one solvent made up of methanol-water (80:20). Dual wavelengths (270 and 350 recommended for Phenolics) were used to detect the eluent composition. The detection was carried out using a photodiode-array UV detector. Extraction efficiency was estimated by the peak area.

## RESULTS AND DISCUSSION

In this experiment the protocol for extracting phenolics were developed by assessing the performance of solvent mixtures and extraction methods. The typical HPLC chromatogram of the 20% methanolic extract is shown in Figure 1. This indicates that *B. coriacea* extract has two major components, peak 1 and peak 2.  Peak 1 component has 53.48 % of the total peak area while peak 2 has about 45.53% of the total peak area. The rest of the minor phenolic components amounted to just 0.99% of the total peak area. This result confirms the earlier report of Ajaiyeoba et al. (2003) that *B. coriacea* has two main components isolated as lupeol and β-sitosterol. Umeh (1991) and Sofowara and Beaaver (1986) also reported the isolation of  β-sitosterol from the plant seed.

## Comparison of the extraction efficiency of solvent composition

The performance of the different solvent mixtures on the total phenolics as evaluated by FC assay is shown in Figure 2. In order to evaluate the impact of solvent polarity on the solubility of phenolics, a systematic extraction approach using the five commonly recommended solvents for phenolics (Luthria and Mukhopadhyay, 2006) were used. The solvents used include methanol, acetone, ethanol and ethyl acetate at 20, 40, 60, 80 and 100% in water. As can be noted from
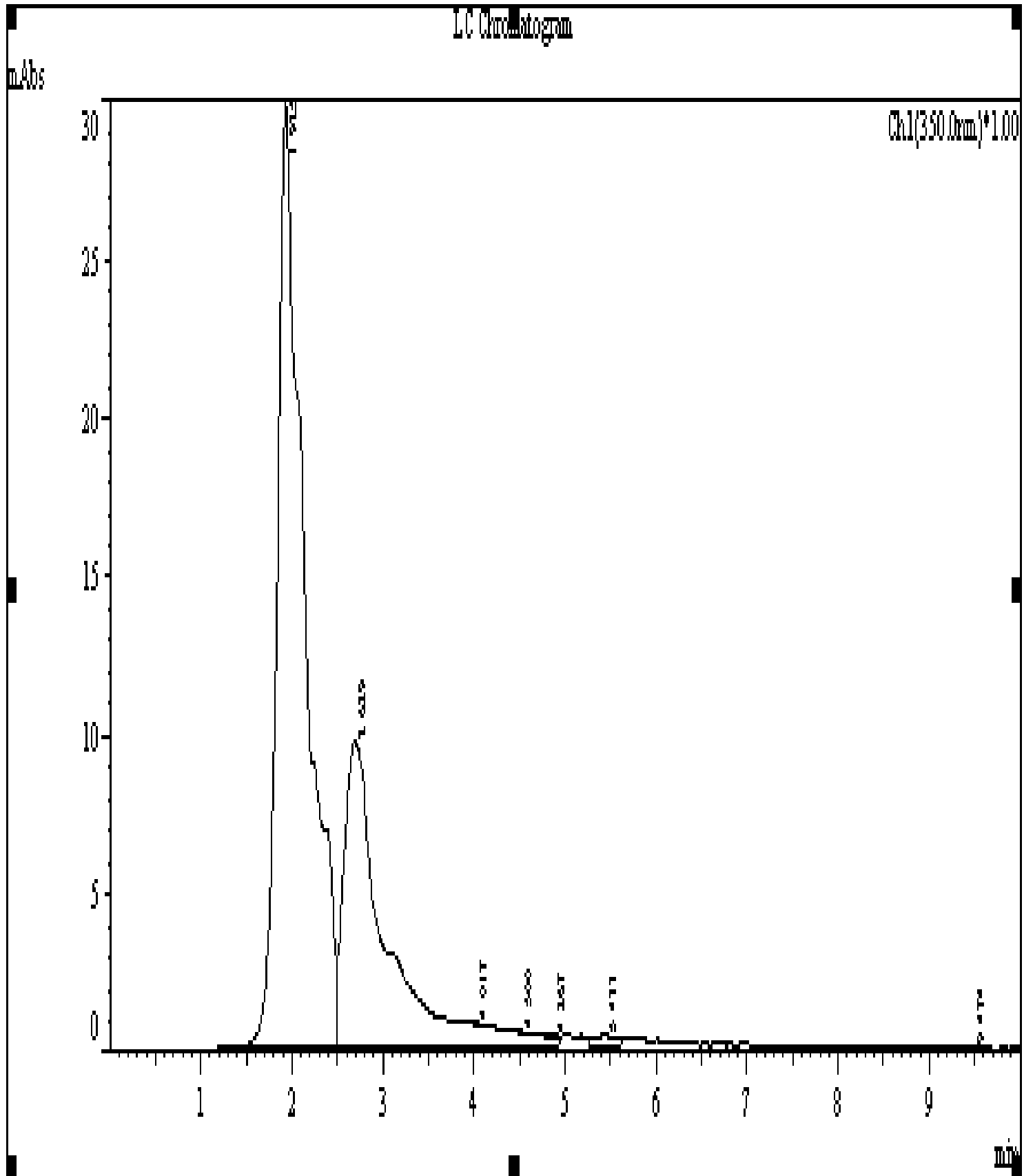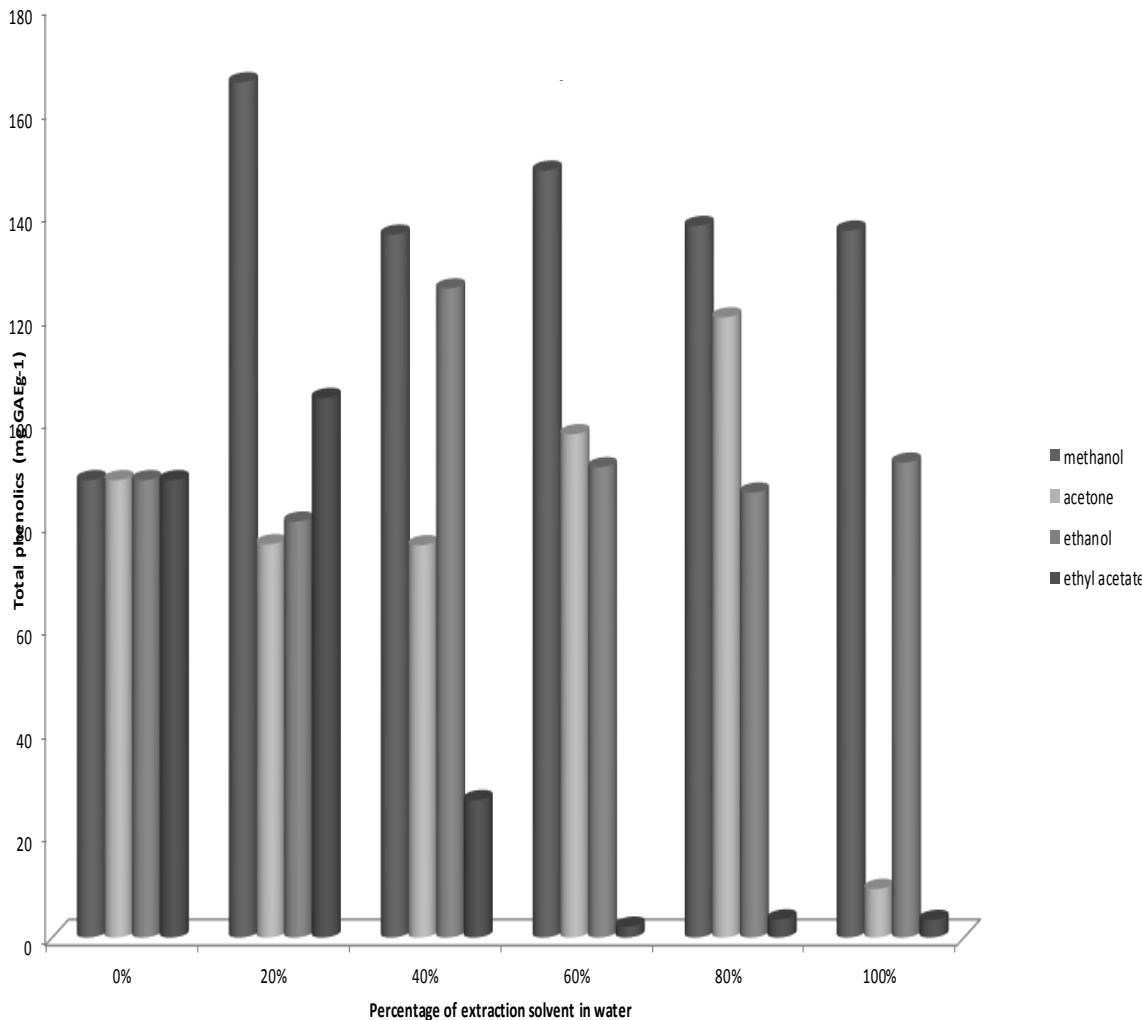
**Figure 1.** The HPLC profile of 20% methanolic extract of *B. coriacea.*

**Figure 2.** Extraction efficiency of phenolic compounds as influenced by solvent composition.

the Figure 2, methanol consistently performed better than any of the other solvents at any rate, yielding the best result at 20% aqueous solution (165 ppm). This was closely followed by ethanol at 40% aqueous solution and acetone at 80% aqueous solution yielding 125.6 ppm and 120.0 ppm respectively. In terms of extraction efficiency (if the 20% methanol that gave the maximum yield is assumed to give 100% efficiency) the 40% ethanol will have efficiency of 76% while the 80% acetone has extraction efficiency of 72%. The least performed solvent was ethyl acetate especially at 60% in water (giving as low as 1% extraction efficiency). The result therefore shows that methanol is the most efficient method of extracting the phenolics in *B. coriacea* and more effective at 20% aqueous solution. This extraction efficiency trend of the various solvents can be explained by the nature of the phenolics and polarity of the solvents. According to Rong and Zeyuan (2004) polar phenolic compounds usually respond more to aqueous

polar solvents in extraction whereas non- polar phenolics are better extracted with non-aqueous solvents. In this case methanol being more polar than the rest of the solvents with a dielectric constant of 33 (Lowery and Richardson, 1987) is expected to perform better than the ethanol (dielectric constant of 30), acetone (dielectric constant of 21) and ethyl acetate (dielectric constant of 6). The outcome agrees with the earlier assertion of Rong and Zeyuan (2004) that methanol is more frequently used than ethanol due to its higher extraction efficiency for polar phenolics. They further emphasized that aqueous methanol between 50 and 80% has been used for extracting hydroxycinnamic acids, and many subgroups of flavonoids. Higher water composition in the solvent can aid in the extraction of glycosides of these compounds.

On the other hand, the HPLC quantitative evaluation of the peak 1 area and peak 2 area as affected by the extraction efficiencies of the different solvents are shown
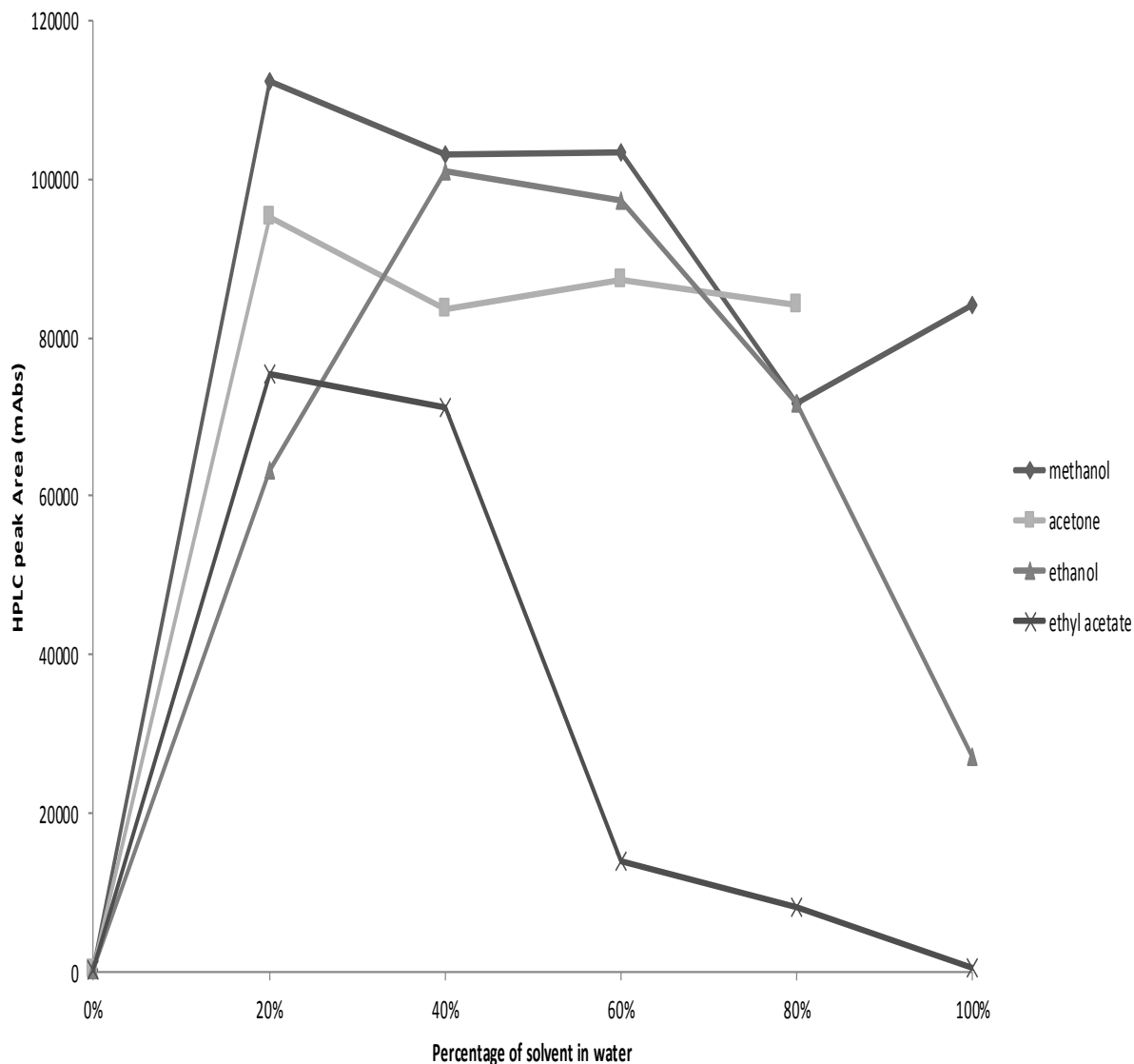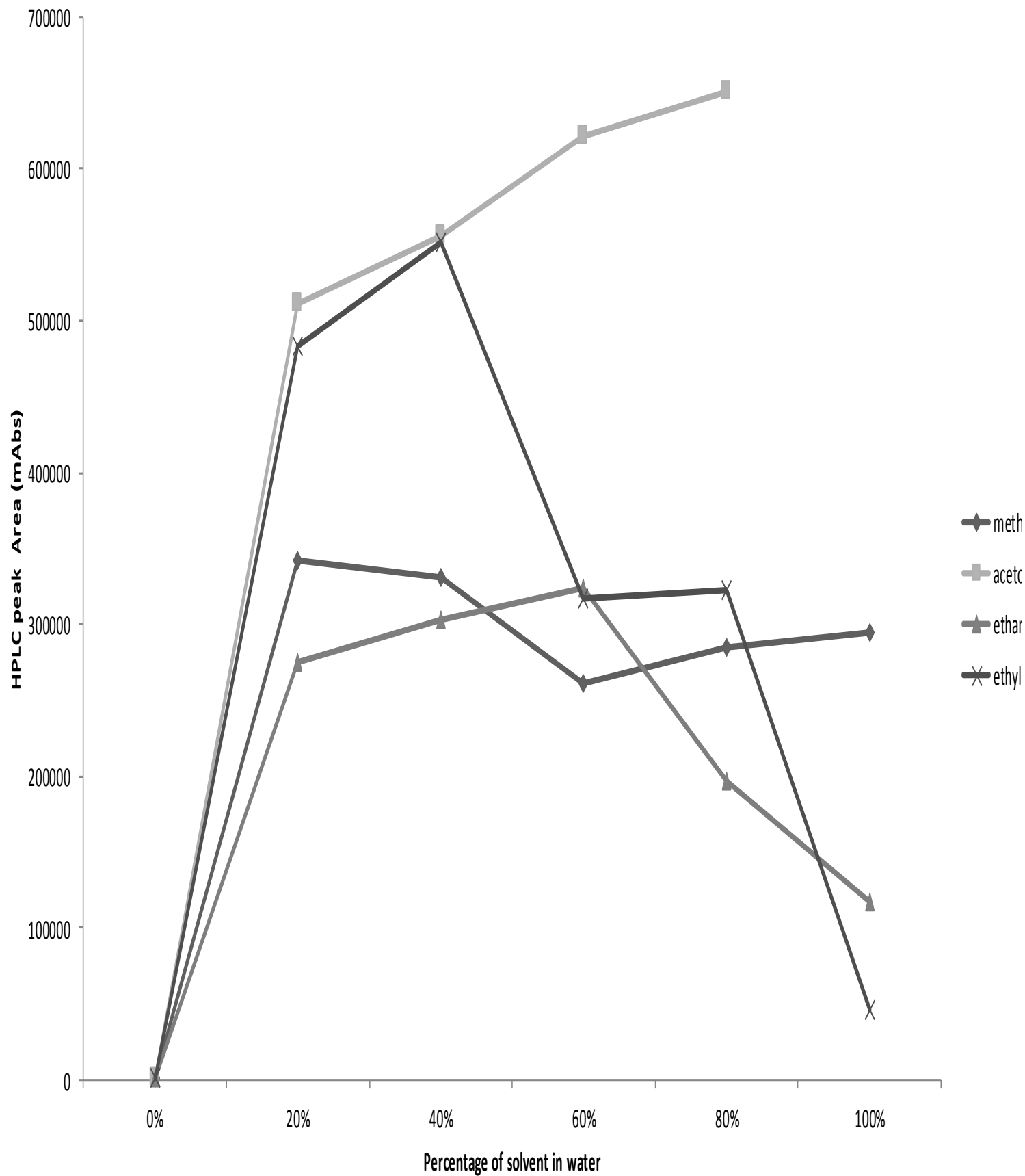
**Figure 3.** influence of extraction solvent on peak 1 component of *B. coriacea.*

in Figures 3 and 4. Again methanol has shown to be most effective amongst all solvents in terms of extracting the peak 1 component of the phenolics (112238.5 mAbs) with 20% aqueous giving the best (Figure 3). This was followed by ethanol (at 40%) and then acetone (at 20%) with extraction efficiencies of 90% and 85% respectively. The least performance was noted with the ethyl acetate (at 100%) with extraction efficiency of 0%. In the case of peak 2 (Figure 4) the most effective solvent was found to be acetone at 80% aqueous solution (651158.5mAbs) followed by ethyl acetate at 40% with extraction efficiency of 85%. Both methanol (at 20%) and ethanol (at 60%) were very poor in extracting the peak 2 component with extraction efficiencies of 53% and 49% respectively. This result obviously shows that the component of peak 1 is more of polar phenolics whereas peak 2 is more of non-

polar in nature. The above results indicated that extraction solvent plays a critical role in the composition of the extract and analyte recovery.

**Comparison of extraction procedures**

The performance of the various extraction methods in terms of the total phenolics (TP) are given in Figure 5. The most effective method was obtained with the use of ASE (136 ppm), followed by the stirring method (135 ppm), then vortex/sonicator (134.1 ppm). That shows that stirring method had 99% extraction efficiency as well as vortex/sonicator. The least efficiency was noted with shaking method (110 ppm) with extraction efficiency of 81%. This result is in agreement with the report of Luthria

**Figure 4.** Influence of extraction solvent on peak 2 component of *B. coriacea.*
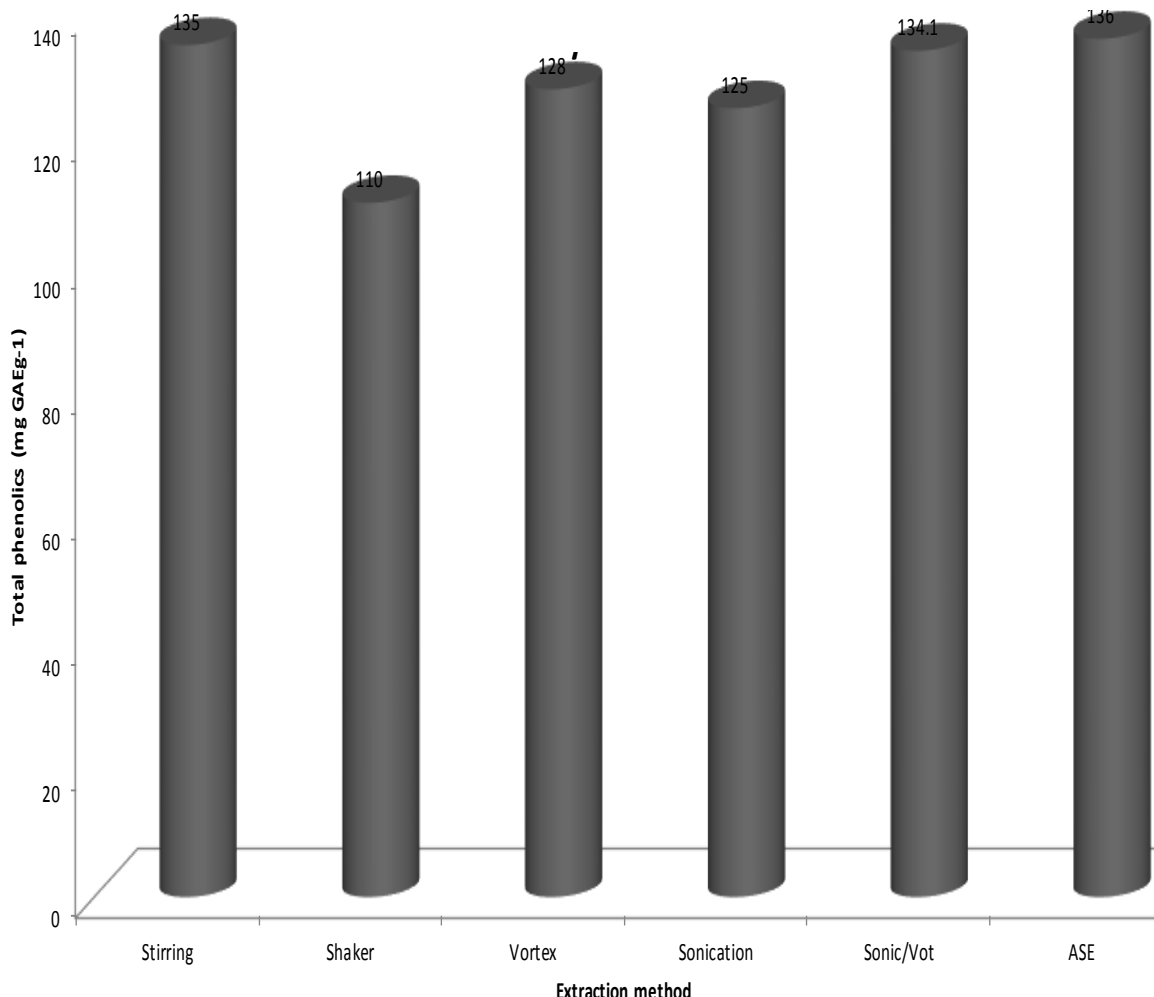
**Figure 5.** Extraction efficiency of total phenolic compounds in *B. coriacea* as influenced by extraction method.

and Mukhopadhyay (2006) who conducted a similar phenolic extraction worked on parsley (*Petroselinum crispum*). They indicated that ASE provided a marginal increase in extraction yields of TP as compared with four other methods (shaking, sonication, stirring and vortex mixing).

On the other hand, the extraction efficiencies of the methods as assessed by the HPLC peak area are shown in Figure 6. It could be noted from the results that the highest efficiency for peak 1 was demonstrated by the combined sonicator/vortexing method, yielding as high as 730766 mAbs, closely followed by the stirring method (697143.5 Abs). The least efficiency for peak 1 component was found to be the one obtained with the use of the ASE, yielding as low as 44743.5mAbs. In terms of extraction efficiency for peak 1, stirring method had efficiency of 95% while, vortexing, shaker, sonicator, and ASE extraction methods had 83, 81, 47 and 6% respectively. On the other hand, the most effective method for extracting peak 2 component was found to be

with the ASE extraction method, which produced as high as 334128.5 mAbs while the shaker method (145172 mAbs) proved to be the weakest extraction protocol. Sonicator method produced efficiency of 91%, stirring 48%, sonicator/vortexing 46%, vortexing 45% while shaker had barely 43% extraction efficient.

It does appear that intensity of agitation as well as environmental condition prevailing during the extraction, influence the extraction efficiency. Thus the high extraction efficiency of ASE procedure can be ascribed to the extraction principles carried out at 40°C under 1000 psi in an inert nitrogen atmosphere. Also the relative high yield under the combined sonicator/vortexing method should be as a result of the level of agitation achieved both under sonication and vortexing which would be more intense than when a single operation is used. Minimal agitation is achieved in shaker, no wonder it had the lowest extraction efficiency. The poor performance of the shaker method also agrees with the report of Luthria and Mudhopadyhy (2006).
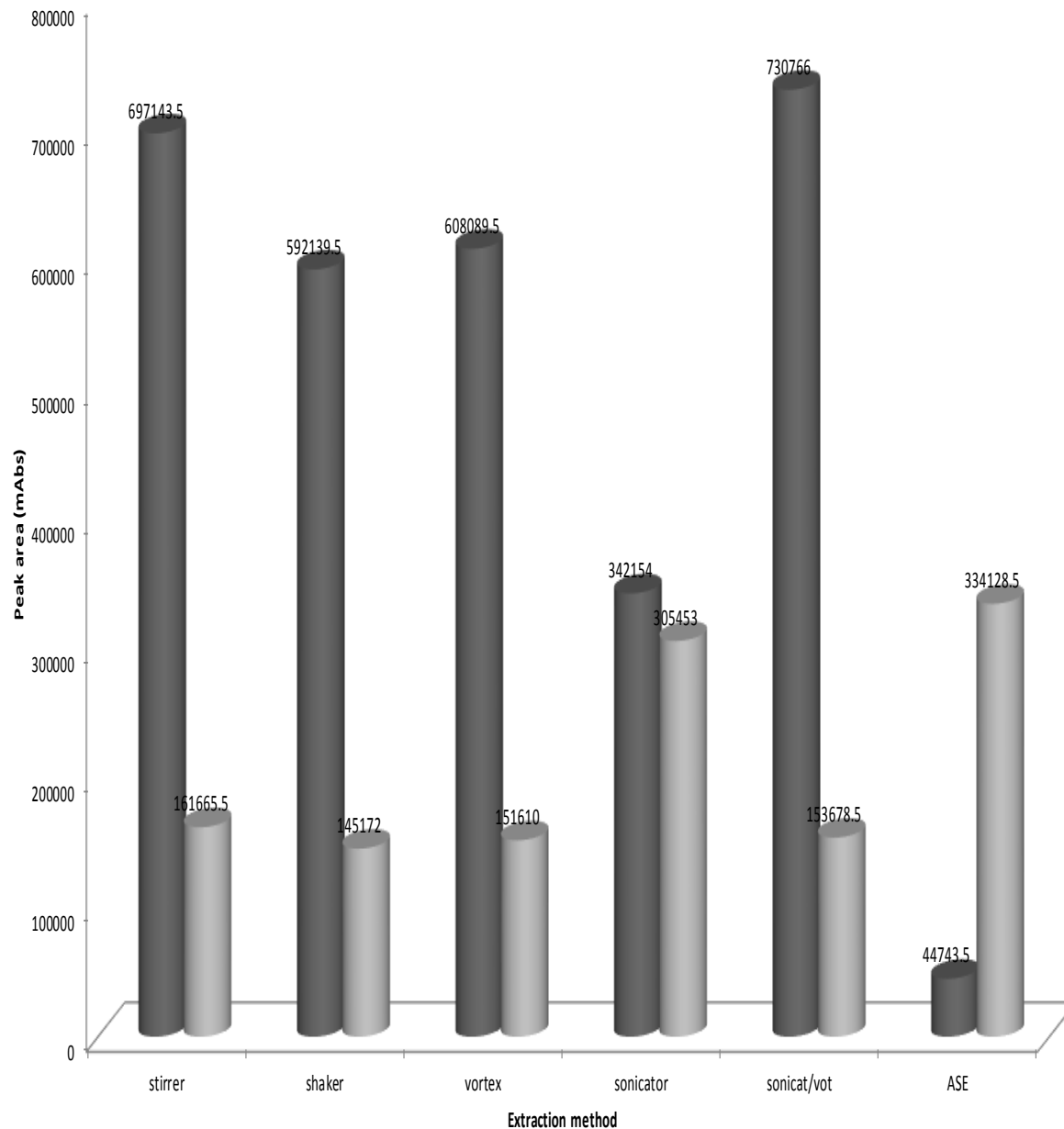
**Figure 6.** Extraction efficiency of peak 1 and 2 component of *B. coriacea* as influenced by extraction method.

## Conclusion

In this experiment two major phenolic compounds peaks (one and two) were isolated from the *B. coriacea* seed extract in the HPLC which were suspected to be lupeol and β-sitosterol. Methanol at 20% aqueous solution was found to be most efficient in extracting the total phenolics and peak 1 component whereas acetone at 80% aqueous solution was more efficient in extracting peak 2 component. On the other hand ASE extraction procedure was more efficient for extracting the total phenolics and peak 2 component while sonicator/vortexing was more efficient in extracting peak 1 component.

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